Studies on *Cenococcum graniforme*. I. An efficient method for isolation from sclerotia

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The mycorrhizal fungus *Cenococcum graniforme* can be readily isolated from sclerotia that have been brushed clean and immersed in 30% H_2O_2 for 10 to 40 minutes. Sclerotia are obtained from small soil samples wetted to a slurry that is swirled to remove the lightweight, sclerotia-containing fraction in a gold-panning type of process. Viable sclerotia sink in water whereas nonviable ones float, so the latter are easily eliminated. Sclerotia have been observed to form in agar cultures, especially those that have been allowed to desiccate slowly for 2 to 3 months.

Introduction

Cenococcum graniforme (Sow.) Ferd. & Winge has attracted much research interest, not only because of its wide distribution as an ectomycorrhizal fungus (Trappe 1964; Trappe and Salinas Quinard 1966) but also because of its production of a water-insoluble antibiotic (Krywolap and Casida 1964; Krywolap *et al.* 1964). Although occurring in forest soils as a sterile mycelium, it is readily identifiable by the combination of distinctive sclerotia, jet black hyphae, and unique mantle structure that it forms on mycorrhizae (Mikola 1948*a*).

Attempts to isolate *C. graniforme* from mycorrhizae by various techniques have always yielded low success (Hatch 1934; Mikola 1948*b*; Zak and Marx 1964). My 193 attempts, using the technique and apparatus of Slankis (1958) but trying various lengths of sterilization time, produced no isolates of the fungus.

Isolation of *C. graniforme* from sclerotia, in contrast, has proved easy. Live sclerotia vary from 0.05 to 4 or more mm in diameter (Fig. 1). Viable, mature ones are jet black, hard, smooth, mostly spherical, often with emergent hyphae, denser than water, and have a high content of ethanol-soluble oil. They are nearly always abundant in soils containing *Cenococcum* mycorrhizae. Dead sclerotia, which can persist indefinitely in soil, look much like live ones but float in water and lack the ethanol-soluble oil.

Although *Cenococcum* sclerotia are a good source for isolation of the fungus, their small size and dark color can make them difficult to find. From several years' study of this fungus in soils and in vitro, however, I have developed simple and efficient methods for obtaining sclerotia, separating nonviable from viable ones, and surface-sterilizing them.

Obtaining Viable Sclerotia

Small soil samples (25–50 cc) are taken from the lower A_0 or the A_1 soil horizons. Since sclerotia tend to be particularly abundant near *Cenococcum* mycorrhizae, mycorrhizal fragments should be included in the sample when convenient. Each sample is put in a small evaporating dish, or one similarly shaped, and wetted to make a slurry with free water on its surface. The dish is then gently hand-swirled by moving it in a small circle, an action which causes the relatively light sclerotia to rise to the top of the heavier soil particles. As swirling continues, the dish is tilted so that a small amount of the slurry edge drops out into a petri dish with each rotation. After about half the slurry has been transferred to the petri dish, the remains can be set aside. The principle is the

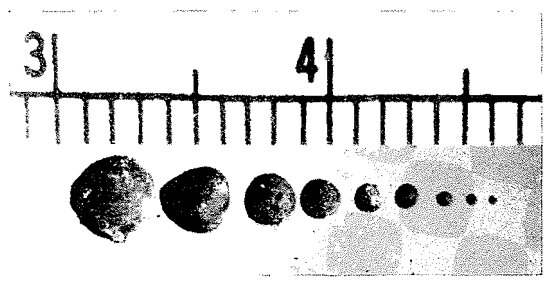

FIG. 1. Sclerotia of *Cenococcum graniforme* from forest soil. Scale in millimeters.

same as used in gold panning, but in this case the lightweight fraction is retained.

The round, black sclerotia in the slurry are easily seen under a dissecting microscope and can be transferred with a forceps to a small container of water. Since large sclerotia remain at the bottom of the evaporating dish despite swirling, the residual fraction can also be examined, if desired. This is quickly done by spreading out the slurry to a depth of 1–2 mm, in which the larger specimens are easily visible to the naked eye. In soils with a large proportion of *Cenococcum* mycorrhizae, a single sample will yield several dozen sclerotia in a few minutes by this method.

Viable sclerotia sink in water when fully imbibed, whereas dead ones float. Those that sink immediately after extraction from soil are ready for surface sterilization. If more are needed from the sample, the floaters can be brushed free of most adherent soil and hyphae under water and left to soak for 12–24 hours. Any that sink during this period are probably viable. Those still floating after 24 hours are dead.

Surface Sterilization and Culturing

Sclerotia selected for isolation of the fungus should be vigorously brushed again to free them of remaining soil and hyphae. They are then immersed in an excess of 30% H_2O_2, at least 5 ml per sclerotium. If the sclerotia have been well cleaned, 10 minutes' immersion gives complete sterilization although as much as 40 minutes does not reduce germination, even of very small specimens. After soaking, they are removed from the H_2O_2 with sterile forceps, blotted on sterile filter paper just enough to leave the surface slightly moist, and transferred without rinsing to a suitable nutrient agar (e.g., Mikola 1948*a*). Pieces of the sclerotia may flake off onto the agar surface from action of the H_2O_2, but this is harmless. Of 60 attempts with this technique, 54 produced pure cultures of the fungus.

The hyphal tips that initially grow from sclerotia, usually within 10 days, are white. As the colony enlarges, older hyphal segments darken. After a month's growth, the oldest hyphae are black macroscopically, and at least some have thick walls microscopically of vinaceous brown to deep purple or even black. The center of older colonies usually develops into a dense, raised, convoluted tissue. These hyphal and colony characteristics are distinctive for *C. graniforme* among associated soil fungi; if only some are present, the identity of the isolate is suspect.

Many isolates produce small sclerotia in agar culture, occasionally within the first month of growth. More often, sclerotia form in 2- to 3-month-old cultures grown at room temperature and allowed to slowly desiccate. As the agar shrinks from the container walls and active colony growth slows, small clusters of rounded cells begin to appear along hyphae. Given the proper conditions, which are unidentified as yet and seem to vary from one isolate to another, these clusters may develop into small, typical sclerotia. Masses of sclerotia also commonly form on the dense, central tissue of older colonies.

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